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14. ABSTRACT
Malignant Pleural Mesothelioma (MPM) survival remains poor despite multidisciplinary treatment involving aggressive surgery, chemotherapy and adjuvant radiotherapy (RT). The large RT treatment volume, and concerns about the proximity of radiosensitive normal structures, restricts the tumoricidal dose of radiotherapy that can be delivered. These concerns limit the effectiveness of adjuvant RT. To overcome this limitation, an entirely novel radiation treatment schedule in combination with post-RT delivery of bone marrow-derived stem cells was examined to improve tumor control and facilitate normal tissue proliferation. A rat model of MPM was used. The RT regime consisted of 10 pulses of low-dose RT (0.2 Gy) using a 3 minute inter-pulse interval (PERT) to a daily dose of 2 Gy. The inclusion of post-RT stem cell therapy is to repopulate normal tissues in the RT field. RT tumor response was assessed by microPET/CT (Positron emission tomography/computed tomography) imaging. In vitro cell survival data was used to demonstrate PERT was not inferior to standard RT (2 Gy single continuous treatments). In vivo, The surgical procedure has been established and tumor model has been established and tumor volume determined by in situ with F18-FDG. Unexpected technological problems with respect to the microPET scanner have slowed the imaging aspect of the project. However, to date, we have demonstrated that RT is effective at reducing MPM tumor growth in vivo; and this is associated with recruitment of hematological stem cells. Studies are currently on-going to determine if PERT is superior to standard RT in MPM.

15. SUBJECT TERMS
Malignant Pleural Mesothelioma, Low dose pulsed radiotherapy, stem cells, animal imaging.

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ABSTRACT

Malignant Pleural Mesothelioma (MPM) survival remains poor. Aggressive surgery, chemotherapy and adjuvant radiotherapy (RT) are often ineffective. The efficacy of the RT component within the multimodality therapy is confounded by the large RT treatment volume. Consequently, radiosensitive normal structures proximal to the tumor are irradiated which restricts the size of the tumoricidal dose of radiotherapy that can be delivered to the tumor. To improve RT effectiveness, we investigated an entirely novel radiation treatment schedule in combination. A rat model of MPM was used. The RT regime consisted of 10 pulses of low-dose RT (0.2 Gy) using a 3 minute inter-pulse interval (PERT) to a daily dose of 2 Gy. RT tumor response was assessed by microPET/CT (Positron Emission Tomography/Computed Tomography) imaging. In vitro cell survival data demonstrated PERT was not inferior to standard RT (2 Gy single continuous treatments). In vivo, the surgical procedure and tumor model were established and tumor volume determined by *in situ* by non-invasive imaging. A significant increase in F18-FDG SUVmax was seen over a five week tumor growth period ($p=0.031$) (BG corrected $p=0.019$). Non-treated orthotopic tumors necessitated animal sacrifice at 42 days due to invasive tumor burden. However, both Pulsed RT and Standard RT produced tumor growth delays, the duration of the growth delay was similar after both modalities ($p=0.863$). Animals were sacrificed at 70 days, but with a lower tumor burden than non-irradiated tumors. This difference in tumor burden sacrifice criteria between non-irradiated and irradiated animals can likely be attributed to daily irradiation and the daily anesthesia that was needed for the irradiation procedure. The hypothesis that discontinuous pulsed RT would be more effective than conventionally-delivered continuous standard radiotherapy to the same total dose, and would also be associated with less normal tissue damage, was not supported by the experimental data. However, Pulsed RT was not inferior to standard RT.

INTRODUCTION AND EXPERIMENTAL CONCEPT

Malignant Pleural Mesothelioma (MPM) survival remains poor despite multidisciplinary treatment involving aggressive surgery, chemotherapy and adjuvant radiotherapy (RT). The large RT treatment volume, and concerns about the proximity of radiosensitive normal structures, restricts the tumoricidal dose of radiotherapy that can be delivered. These concerns limit the effectiveness of adjuvant RT. We propose to deliver the radiotherapy using an entirely novel treatment schedule and combine this with post-RT local-delivery of bone marrow-derived stem cells to facilitate normal tissue proliferation. The concept is to deliver 10 pulses of low-dose RT (0.2 Gy) using a 3 minute inter-pulse interval (PERT) to introduce the RT-induced damage at a level that evades ATM dose-dependent DNA damage detection and repair mechanisms.

This research proposal investigated a novel delivery scheme of pulsed radiotherapy; in which the same total daily dose of radiation (2 Gy) is given as 10 individual 0.2 Gy pulses with a 3-minute inter-pulse interval between each individual 0.2 Gy pulse. The total 2 Gy dose is therefore given using a prolonged discontinuous schedule rather than as one continuous treatment used in conventional delivery. The rationale for giving pulsed treatments is that radiation-induced DNA lesions induced by pulsed radiotherapy occurs at a rate below the detection threshold of cellular repair processes in tumor cells, and thus DNA damage accumulates, causing cell death by apoptosis at the next mitosis.

The novelty of this application was the use of pulsed RT, use of an orthotopic mesothelioma tumor model, image-guided radiotherapy using an Xstrahl Small Animal Radiation Research Platform and non-invasive biological imaging using [18F]fluoro-2-deoxy-D-glucose (FDG) and 3′-[18F]fluoro-3′-doxythymidine (FLT) to define tumor response to treatment.

BODY

The application proposed the use of an orthotopic tumor model. MSTO-211H cells were surgically implanted into NIH-Foxn1rnu (nude rats from Charles River). The initial surgical technique to establish the Malignant Pleural Mesothelioma (MPM) model followed the published technique of Ampollini and colleagues [Ampollini et al. Intrapleural polymeric films containing cisplatin for malignant pleural mesothelioma in a rat tumour model: a preliminary study - European Journal of Cardio-thoracic Surgery 37 (2010)557-565]. Under general anesthesia (1-3% isoflurane breathing), the tumor cell suspension was inoculated with a thin needle (27 gauge) in the muscle surrounding the parietal pleura via a small left-sided incision in the thorax between the fifth intercostal space. The chest wall muscles were re-approximated with sutures, and the skin was closed with absorbable sutures. After implantation, animals were prophylactically treated with Augmentin in drinking water at 0.35 mg/ml concentration for one day before surgery and three days post-surgery. Although this technique provided tumors in the majority of implanted animals, the tissue repair from the surgical incision and suturing was detected by F18-FDG imaging which negated this technique was a method of monitoring tumor response. After consultation with the veterinarian, the implant technique was modified to eliminate the surgical incision and suturing and use palpation to locate the needle at the fifth intercostal space. This change in procedure produced tumors in 65% of the implanted animals but extended the timeframe of the study, under the guidance of the attending veterinarian. Each change to the surgical procedure was achieved using one animal at a time. Consequently, small iterations in the surgical procedure took months to establish since the growth rate of the tumor was slow, and further iterations could not occur until the previous animal had been allowed sufficient time for the tumor to grow and develop, or not. The veterinarian only allowed individual animals to be implanted until the technique was successfully established, as a complex survival-surgery procedure was required; this limited the rate of progress.

However, following implant, tumors developed with 22±14 days of implant, and animals were scanned with PET/CT weekly once tumors were evident.

The modified surgical procedure was established in the new NIH-Foxn1rnu and tumors were imaged *in situ* with F18-FDG and reconstructed using imaging software (Fig. 1). The tumors were evident in the pleural cavity. Once established, the tumors increased in size per week as indicated by the changed in F18-FDG SUVmax reading (Fig. 2). The larger the SUVmax reading, the higher the metabolic rate of the tumor which is a surrogate measure of tumor growth rate. Gross histological assessment of the implanted animals demonstrated evident MPM tumors (Fig. 3).

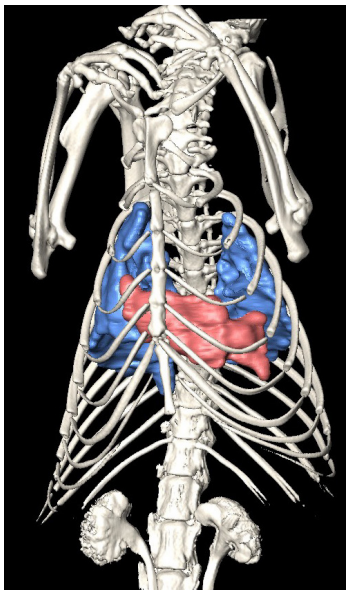

Figure 1: Representative orthotopic mesothelioma tumor. The lungs are pseudo-colored blue and red color indicates the mesothelioma tumor.

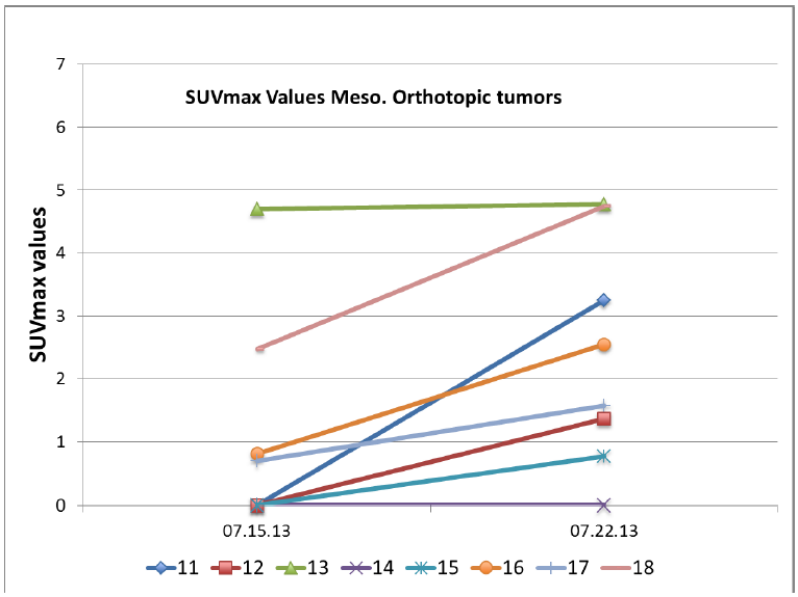

Figure 2: Change in growth rate as indicated by SUVmax for mesothelioma tumors implanted in animals #11-#18. Tumor volume increased over the time frame 07.15.13 to 07.22.13

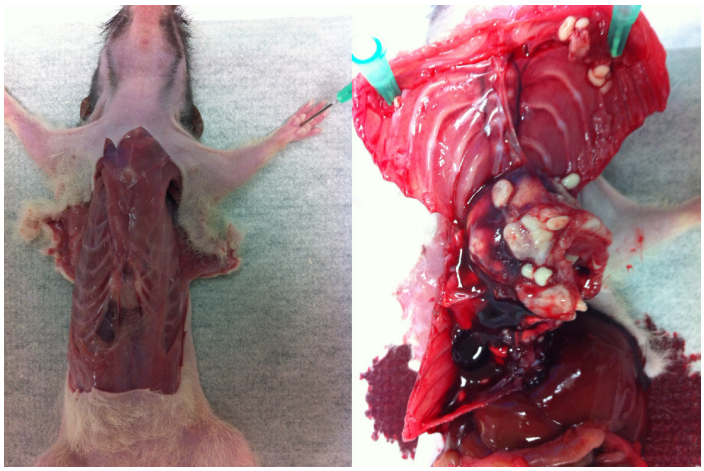

Figure 3: Mesothelioma tumors in the plural cavity and invading the lung.

The growth rate of the tumors and SUVmax values showed a degree of variability between individual animals (Fig. 4). These differences could be normalized by comparing the F18-FDG intensity signal between the tumor-masses and normal tissues within each animal for normalized SUVmax plots (Fig. 5), and to generate a standard (std) and corrected SUVmax value (BG corrected) over the five week period (Fig. 6). The increase in tumor values (blue bars) over the five week period when compared to the limited changes in muscle and heart indicates the tumors are growing.

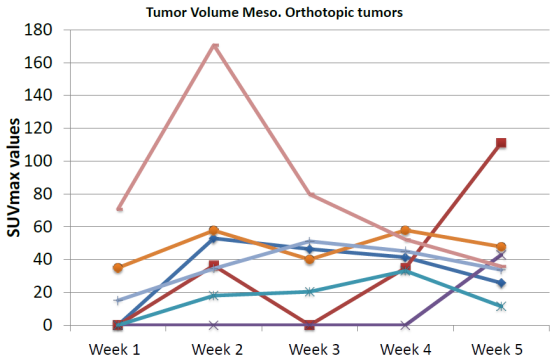

Figure 4: F18-FDG SUVmax over a five week period for seven implanted animals.

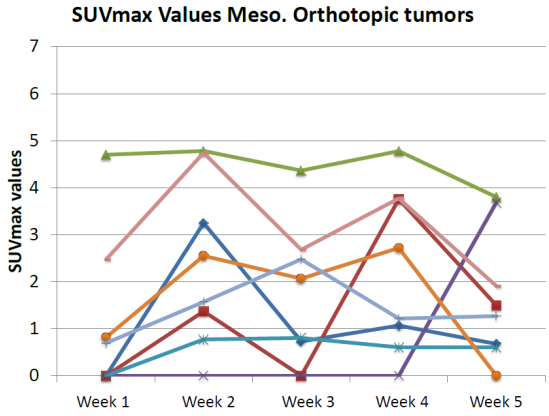

Figure 5: F18-FDG SUVmax over a five week period for seven implanted animals.

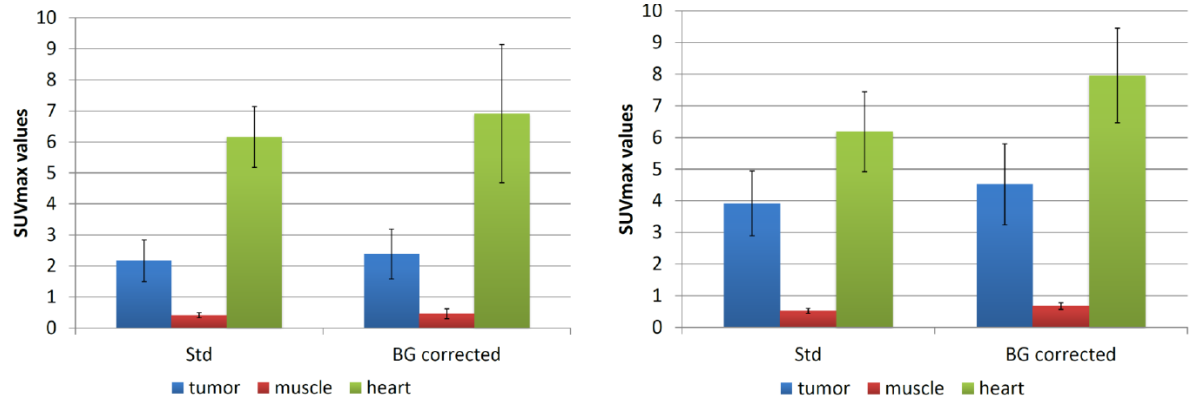

Figure 6: Increase in F18-FDG SUVmax over a five week period. Values are mean±SD for seven implanted animals. There was a significant increase in tumor SUVmax values from the left hand panel (week 1 scans) to right hand panel (week 5 scans). Std p=0.031; BG corrected p=0.019 [Student T-test].

Once the tumor model had been established, the radiation procedure of administering a low-dose pulsed treatment regime using the micro-irradiator was developed. Animals are anesthetized during irradiation procedure (10 doses of 0.2 Gy given daily for 5 consecutive days) with 2-3% isoflurane; this procedure was defined and wass well-tolerated.

Rats were imaged and treated in a vertical position (Figs. 7 and 8). This positional orientation was found to be more effective and reproducible than the prone position as the lung remained extended.

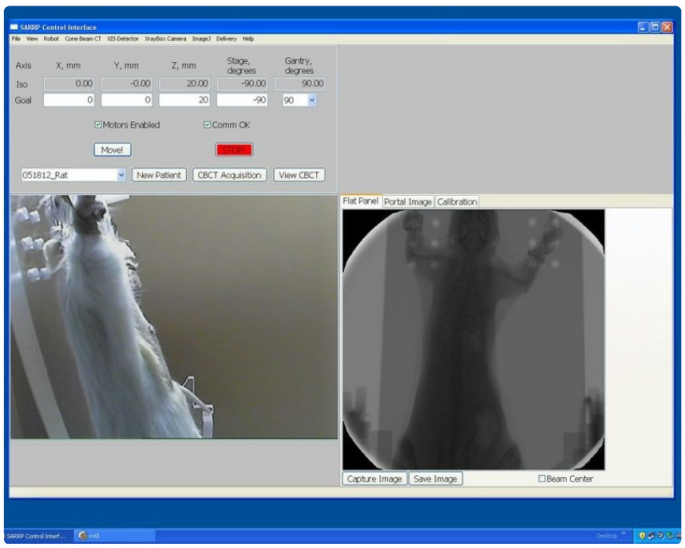

Fig. 8: SARRP graphical user interface with vertical rat bed showing CT image and gantry rotation to achieve 200 cGy dose.

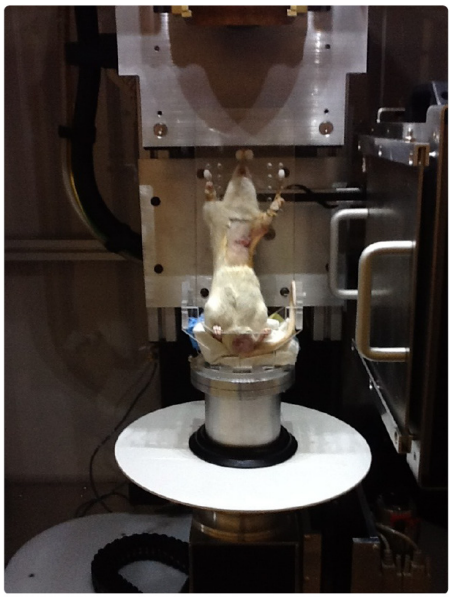

Figure 7: SARRP set-up with vertical rat bed.

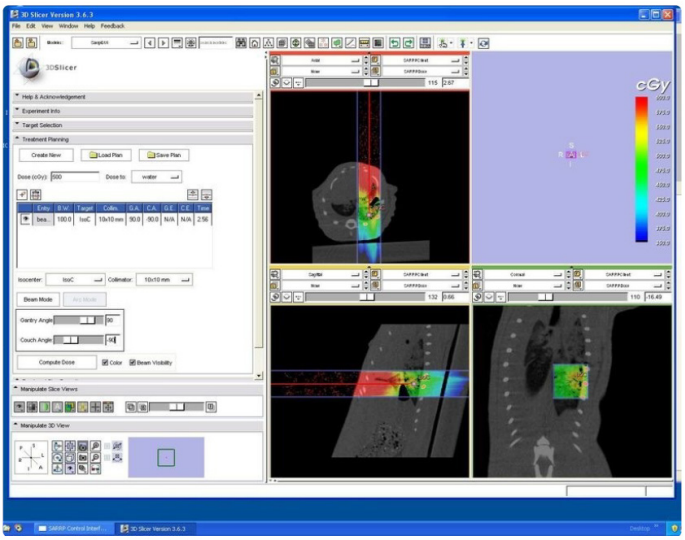

Fig. 9.

Fig. 9. Experimental treatment plan developed to give a single dose of 200 cGy using SARRP-generated CT images in three planes. Treatment plans were developed using the on-board SARRP imaging and planning software. Dose distributions are defined by the colored bar.

A final SARRP treatment plan was finally developed and used to treat orthotopic lung animals with standard 2 Gy treatments and PERT treatments (Fig. 10), this plan was specific for each individual animal as each tumor was unique. Figure 11 indicates anatomical F18-FDG PET and CT imaging; the area of FDG uptake is clearly evident in the lung of the MPM tumor model.

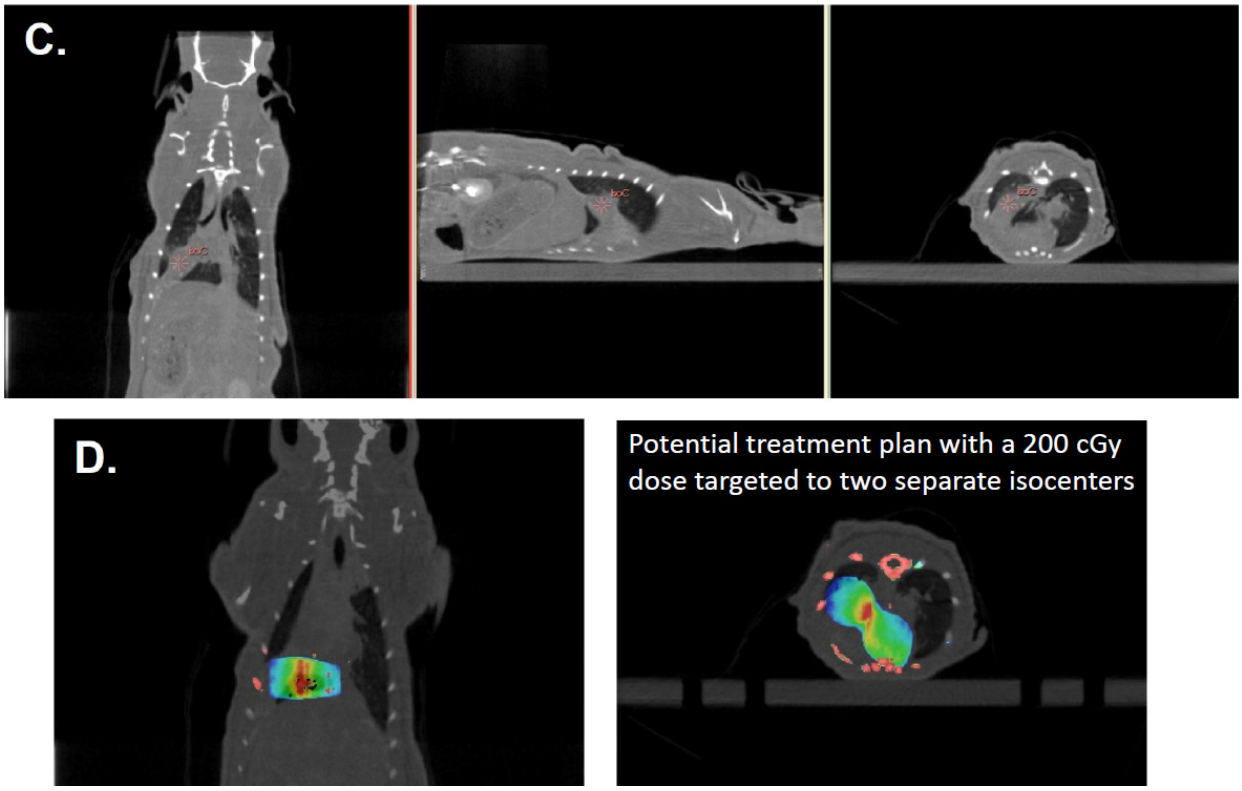

Figure 10. Typical CT image indicating orthotopic mesothelioma tumors (panel C red star) and radiation treatment plan indicating pseudocolored radiation dose. Red color indicates 2 Gy to the tumor volume.

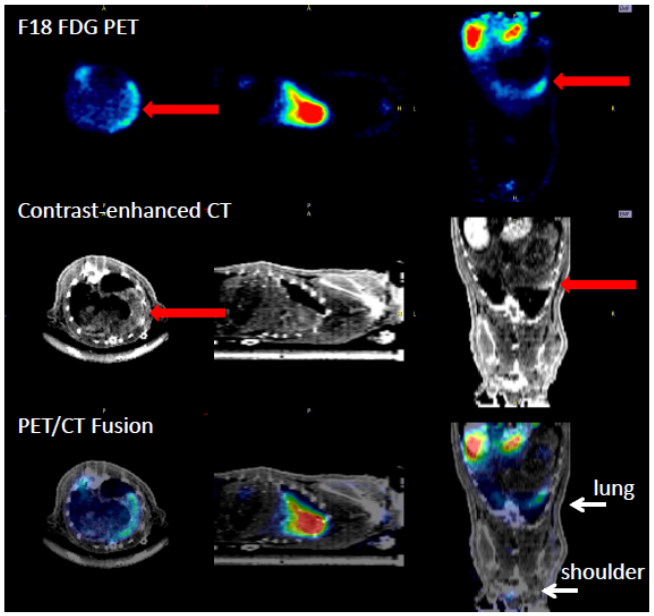

Figure 11. anatomical F18-FDG PET and CT imaging demonstrating uptake in the lung.

EFFECTS OF THE RADIATION TREATMENT.

The radiosensitivity of MSTO-211H cells were initially assayed in vitro using a standard clonogenic assay. 250-350 MSTO-211H cells were plated in T25 flasks and irradiated at 0.69 Gy/min using a 160 kVp Faxitron x-ray cabinet and incubated for 15 days to allow for colony growth. Subsequently, surviving colonies were stained and counted. Surviving fraction was calculated as a function of plating efficiency for non-irradiated cells. The SF2 (surviving fraction at 2 Gy) for MSTO-211H cells after pulsed RT was 0.565 (SD±0.118) and 0.512 (SD±0.089) after standard RT. There was no statistical difference in survival after a single 2 Gy dose between pulsed RT and standard RT. A second mesothelioma cell line NCI-H2052 also demonstrated no difference in survival between pulse RT (SF2=0.702±0.064) and standard RT (SF2=0.637±0.089) after a single dose of 2 Gy. PRT was not inferior to SRT.

Animals were implanted with MSTO-211H cells and tumors were irradiated with 30 Gy in daily 2 Gy factions once tumor growth has been established by non-invasive imaging. Animals were anesthetized with isoflurane for treatment and given either PRT (n=4) or SRT (n=4). Tumor volume was determined by PET/CT. The data in Figure 12 demonstrate that no significant difference was seen between the two treatment regimens (p=0.881). Once established, the tumors grew rapidly and were shown to be radioresistant. Quantitative histological assessment of harvested tissues failed to demonstrate differences in number of proliferating tumor cells (p=0.425) or CD34 stained tumor vessels (p=0.263) in equivalent tumor areas per high power field. No differences in VEGF staining (p=0.635) were seen between the two treatment regiments. The data in Figure 3 indicated that tumor growth was multi-focal and

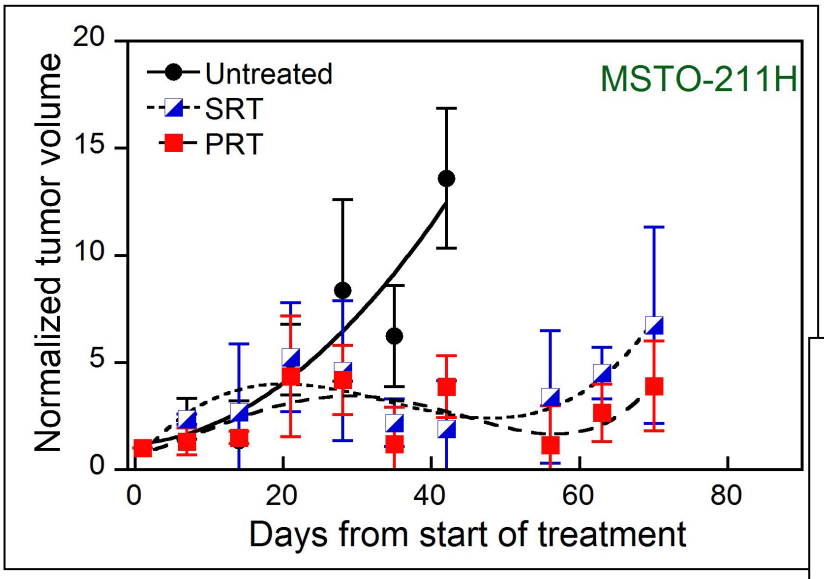

Figure 12. Normalized tumor growth curves for orthotopic mesothelioma tumors treated with Standard RT (SRT) and Pulsed RT (PRT). Untreated animals are shown by data in solid circle. (n=4; mean±SD)

comparative histological assessment proven difficult.

Non-treated tumors necessitated animal sacrifice at 42 days due to tumor burden. However, both Pulsed RT and Standard RT produced tumor growth delays, the duration of the growth delay was similar after both modalities ($p=0.863$). Animals were sacrificed at 70 days, but these animals exhibited a lower tumor burden than non-irradiated tumors. This difference in tumor volume for sacrifice criteria between irradiated and non-irradiated tumors can likely to be attributed to daily irradiation and anesthesia that was needed for the irradiation procedure. Pulsed RT was not inferior to standard RT.

KEY RESEARCH ACCOMPLISHMENTS

- Measure survival response of MPM cells in vitro
- Demonstrated PERT is not inferior to standard RT in vitro
- Established animal model with a change in rat strain to improve tumor reliability
- Demonstrated RT reduces MPM growth in vivo and growth rate in a consistent manner
- Planned treatments on the SARRP
- Treated orthotopic MPM tumors using pulsed and standard RT.
- Evaluated orthotopic tumor growth and treatment response with F18-FDG.

REPORTABLE OUTCOMES AND CONCLUSIONS

The hypothesis was that discontinuous pulsed RT would be more effective than standardly-delivered continuous radiotherapy to the same total dose, and provide less normal tissue damage. The rationale being that radiation damage produced by pulsed RT occurs below the threshold to activate DNA repair mechanisms and therefore radiation-induced damage would remain unrepaired post-treatment and produce a post-mitotic cell death. By contrast, radiation-induced damage from continuously delivered standard RT would produce suffice damage to activate DNA repair mechanism and therefore would prove be less effective than PRT.

The presented data do not support this hypothesis.

The tumor imaging and radiation treatment delivery was presented as a component of the research paper at the World Molecular Imaging conference by Dr. Sarah Krueger.

FUTURE EXPERIMENTS

The aim of future experiments, should funding be obtained, is to combined pulsed RT with cytotoxic or targeted chemotherapeutic agents to provide better tumor control.

REFERENCES AND APPENDICES

None. Data is still being compiled for publication.

WITH RESPECT TO STATEMENT OF WORK

Specific Aim #1 – Establish and treat Mesothelioma model (Months 1-7)
Overview. The aim is to develop Mesothelioma model and treat with pulsed radiotherapy.

Subtask1: Establish surgical technique and tumor implantation (Months 1-2)

a. Purchase and acclimatize 12-week old rats.
b. Establish surgery procedure for intrapulmonary implanting of Mesothelioma cells.
c. Ensure successful infection-free surgery without adverse pulmonary breathing rates or events.

The work from Subtask1 is complete.

Subtask2: Compare Radiation schedules (Months 2-7)

a. Measure breathing rate and lung function in tumor-bearing animals
b. Obtain weekly blood samples
c. Treat with conventional fractionated radiotherapy and pulse schedules
d. MicroPET scan animals to assess treatment outcomes
e. Harvest lungs and other tissue for histopathology
f. Analyze data set to determine effectiveness of two RT schedules.

The work from Subtask2 is complete for untreated animals. Task f have not been completed but is underway.

Subtask3: Compare Radiation schedules (Months 2-10)

g. Measure breathing rate and lung function in tumor bearing animals
h. Obtain weekly blood samples
i. Treat with conventional fractionated radiotherapy and pulse schedules ***plus stem cell therapy***
j. MicroPET scan animals to assess treatment outcomes
k. Harvest lungs and other tissue for histopathology
l. Analyze data set to determine effectiveness of two RT schedules.

Tasks g, h, k, have been completed for untreated control animals. Task i is not complete but stem cells have been successfully harvested and given to recipient animals.

Specific Aim #2 – Analysis microPET images and compare with histology (Months 10-12)
Overview. Compare outcomes of difference RT schedules in the presence and absence of stem cells.

Specific Aim #2 examines excised tumors using histopathology and compares with microPET analysis (Months 10-12)

Subtask1: Surgically excise tumor (regrowth) and normal tissues from treatment animals.

a. Surgically extract intrapulmonary tumors
b. Block and section tissue for histological examination
c. Cut sections and stain with H&E and specific immunochemistry

Subtask2: Compare histology with non-invasive tumor imaging (Months 3-12)

a. Mathematically compare **histology** with functional SUV imaging data

b. Analyze entire data set to determine if PERT is more effective than conventional RT for tumor regrowth and the outcome of stem cell therapy, as confirmed by histology and microPET/CT imaging.

The work from Aim 2 was almost completed for unirradiated and irradiated animals. We were unable to complete the stem cell aspects of the project. Sub-tasks are fully complete for animals given standard RT or PERT, but without he stem cell experiments to repair normal tissues. No significant differences were seen in a range of histology parameters between standard or pulsed RT.